Plant diversity increases N removal in constructed wetlands when multiple rather than single N processes are considered

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Abstract: Biodiversity has a close relationship with ecosystem functioning. For most biodiversity–ecosystem-functioning studies, biodiversity has been linked to a single indicator variable of ecosystem functioning. However, there are generally multiple ecosystem processes contributing to ecosystem functioning and they differ in their dependence on biodiversity. Thus, the relationship between biodiversity and ecosystem functioning can be stronger when multiple rather than single ecosystem processes are considered. Using both mass-balance and stable-isotope approaches, we explored the effects of plant diversity on nitrogen (N) removal sustained by multiple N-cycling processes in experimental microcosms simulating constructed wetlands, an ecosystem treating wastewater with high N loading. Four species were used to assemble different plant communities, ranging in richness from one to four species. The removal of N, indicated by low levels of total inorganic N concentration (TIN) present in the effluent, was considered as an integrated measure of ecosystem functioning, combining three constituent N-cycling processes: plant uptake, denitrification, and substrate adsorption. Our results showed that (1) species richness had a positive effect on N removal, in particular, the four-species mixture reduced effluent TIN to a lower level than any monoculture; however, polycultures (two-, three-, and four-species mixtures) did not outperform the most efficient monoculture when each of the three constituent N-cycling processes was considered by itself; (2) species identity had significant impacts on single processes. Communities with the species Coix lacryma-jobi showed the greatest capacity for N uptake and communities with Phragmites australis had the highest denitrification rates; (3) isotope fractionation in the rhizosphere of Coix lacryma-jobi was primarily due to microbial denitrification while multistep isotope fractionation was detected for Phragmites australis and Acorus calamus (indicating recycling of N), suggesting that species differed in the way they transformed N; (4) the enhanced N removal at high diversity may be due to mutualistic interactions among species belonging to different functional types. Our findings demonstrated that although plant species richness had negligible effects on individual N-cycling processes, it enhanced the overall ecosystem functioning (N removal) when these processes were considered collectively. Our study thus contributes to improve the treatment efficiency of constructed wetlands through proper vegetation management.

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Plant diversity increases N removal in constructed wetlands when multiple rather than single N processes are considered

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Abstract

Biodiversity has a close relationship with ecosystem functioning. For most biodiversity–ecosystem functioning studies, biodiversity has been linked to a single indicator variable of ecosystem functioning. However, there are generally multiple ecosystem processes contributing to ecosystem functioning and they differ in their dependence on biodiversity. Thus, the relationship between biodiversity and ecosystem functioning can be stronger when multiple rather than single ecosystem processes are considered. Using both mass-balance and stable-isotope approaches, we explored the effects of plant diversity on nitrogen (N) removal sustained by multiple N-cycling processes in experimental microcosms simulating constructed wetlands, an ecosystem treating wastewater with high N loading. Four species were used to assemble different plant communities, ranging in richness from one to four species. The removal of N, indicated by low levels of total inorganic N concentration (TIN) present in the effluent, was considered as an integrated measure of ecosystem functioning, combining three constituent N-cycling processes: plant uptake, denitrification and substrate adsorption. Our results showed that: (1) species richness had a positive effect on N removal, in particular, the four-species mixture reduced effluent TIN to a lower level than any monoculture of the component species, however, polycultures (two-, three-, and four-species mixtures) did not outperform the most efficient monoculture when each of the three constituent N-cycling processes was considered by itself; (2) instead, species identities had significant impacts on single processes. Communities with the species *Coix lacryma-jobi* showed the greatest capacity for N uptake and communities with *Phragmites australis* had the highest denitrification rates; (3) isotope fractionation in the rhizosphere of *Coix lacryma-jobi* was primarily due

to microbial denitrification while multistep isotope fractionation was detected for *Phragmites australis* and *Acorus calamus* (indicating recycling of N), suggesting that species differed in the way they transformed N; (4) the enhanced N removal at high diversity may be due to mutualistic interactions among species belonging to different functional types. Our findings demonstrated that although plant species richness had negligible effects on individual N-cycling processes, it enhanced the overall ecosystem functioning (N removal) when these processes were considered collectively. Our study thus highlights the complementarity across multiple processes to sustain ecosystem functioning and contributes to improve the treatment efficiency of constructed wetlands through proper vegetation management.

Key words: biodiversity, denitrification, microcosm experiment, N cycling, plant uptake, species identity, species richness, substrate adsorption

INTRODUCTION

Biodiversity–ecosystem functioning (BEF) relationships have been widely investigated, both experimentally and theoretically (e.g., Tilman et al. 1997, Hector et al. 1999, Brose and Hillebrand, 2016). These BEF studies have systematically explored the resource partitioning among competing species and plant–plant interactions. In addition, comparison of the performance of species mixtures with monocultures has been recognized as another important tool in the assessment of biodiversity effects. For example, a phenomenon called "over-yielding" suggests that the biomass production of a mixture is greater than the average monoculture biomass of the component species (non-transgressive overyielding; e.g., Hector et al. 2002, Hooper and Dukes 2004) or even greater than the most productive monoculture (transgressive overyielding; e.g., Loreau 1998, Schmid et al. 2008). Similarly, when species mixtures deplete more nutrients, such as nitrogen (N) in soil or water than the single most efficient species, transgressive "under-depletion" occurs (Palmborg et al. 2005, Cardinale 2011). Moreover, the effects of species diversity on resource depletion have been shown to vary under different levels of nutrient supply (Fridley 2002, 2003, Isbell et al. 2013, Siebenkas et al. 2016).

Studies about the effects of species diversity on N depletion have been, so far, overwhelmingly based on experimental approaches in N-limited environments (plant growth is often limited by N availability), such as grassland ecosystems, where the depletion of soil N largely results from N sequestration by plants (Fornara and Tilman 2009). In contrast, in riparian wetlands and constructed wetlands (CWs) with high N supply, the proportion of plant consumption of N may be lower while other outflow pathways for N, such as denitrification and leaching, may be higher than in grassland

ecosystems (Hocking and Reynolds 2011). For example, Bachand and Horne (2000) demonstrated that denitrification rather than plant uptake was the main mechanism for nitrate removal in six CWs located at the upstream of Santa Ana River in California. In this case, the use of a single response variable (plant N uptake) as proxy for the integrative ecosystem functioning (i.e., N removal) may be problematic due to the omission of other important ecosystem processes (Gamfeldt et al. 2008).

Recent studies have demonstrated that a major functional importance of biodiversity is to sustain multiple ecosystem processes and functions (Isbell et al. 2011, Perkins et al. 2015, Meyer et al. 2018, Le Bagousse-Pinguet et al. 2019). However, the majority of BEF studies have been on bivariate relationships between biodiversity and a single ecosystem process (e.g., Isbell et al. 2013, Abalos et al. 2014, Lange et al. 2015). Because species differ in their ecology and physiology and therefore their contributions to different biogeochemical processes, the effects of plant diversity on individual processes and on integrated ecosystem functioning, combining multiple processes, may be fundamentally different (Gamfeldt et al. 2008, Perkins et al. 2015). The N removal in a N-rich ecosystem (e.g., a CW) can be considered as representative of such integrative ecosystem functioning, because several ecosystem processes contribute to the total amount of N removal, in particular plant uptake (with subsequent harvest), denitrification and substrate adsorption (Vymazal 2007). It is conceivable that plant diversity effects work in different ways via the single processes to yield the integrated ecosystem functioning. Thus, the effects of plant diversity on both single N processes and integrated N removal should be considered.

CWs have been widely applied for treating wastewater with a high level of N loading. Previous studies have observed that in CWs high plant species richness could enhance N removal from wastewater (Fraser et al. 2004, Maltais-Landry et al. 2009, Zhang et al. 2010). Nevertheless, the mechanisms underpinning this effect are not well understood. Over the past two decades, small-scale microcosm experiments have been initiated to simulate the full-scale CWs and test plant diversity effects on nutrient removal (Tanner 1996, Picard et al. 2005, Ge et al. 2015). The confined microcosm systems and readily measurable parameters allow us to quantify the amount of N removed from the system as well as N contents in various compartments of the system (influent, effluent, plant tissues and substrate). However, due to the high complexity associated with N transformation in CWs, the commonly used mass-balance approach, based on the net change in nutrient contents, cannot identify the N cycling routes in microcosms. Investigating the change in isotope signature between influent and effluent variables could provide information on each N transformation (production and uptake processes of different N forms). The coupling of nutrient and isotope flux measurements has been reported in CWs (Reinhardt et al. 2006, Sovik and Morkved 2008, Erler and Eyre 2010), but the use of this technique in BEF research has not, to our knowledge, been attempted.

In this study, we used a microcosm experiment simulating CWs to quantify the rates of N removal and three key processes contributing to N removal (plant uptake, denitrification and substrate adsorption) in response to changes in plant diversity (plant species richness, identity and composition). The N input into each microcosm was controlled by supplying simulated wastewater with defined concentrations and forms of N. The total inorganic N (TIN, the sum of nitrate and ammonium) present in the effluent

was used to measure the amount of N being removed. Both a mass-balance approach (MBA) and a stable-isotope approach (SIA) were used to quantify N removal as well as to trace N cycling in the microcosms, respectively. Our objectives were to examine the effects of: (1) plant species richness and identity on N removal and its three constituent N-cycling processes (plant uptake, denitrification and substrate adsorption); (2) plant species on N transformation and cycling within the microcosms; and (3) species composition on the coupling of different constituent N-cycling processes through species interactions and coexistence. Predicting multifunctional complementarity among species (species are complementary when different species dominate different processes; see e.g., Hector and Bagchi 2007), we tested whether plant diversity becomes more important in sustaining a high level of N removal when multiple constituent N-cycling processes are considered jointly, even though individual processes may not be enhanced in mixtures of plant species.

MATERIALS AND METHODS

Principle and methodology

Because plant species selected for wastewater treatment in CWs are generally herbaceous, we compare CWs with grassland ecosystems for major N flows in these two ecosystems. Effects of plant diversity on the plant N pool and the depletion of soil N (the grassland ecosystem) or wastewater N (the CW ecosystem) are illustrated in Fig. 1. Four classes of responses are involved: (i) with increasing diversity the amount of N accumulated in plant biomass increases and the degree of soil-N depletion (or wastewater N removal) also increases (Fig. 1a-i, 1b-i). This may occur when the reduction of resource

154 is primarily caused by plant uptake (Palmborg et al. 2005, van Ruijven and Berendse
155 2005). (ii) The N abundance in soil or wastewater does not decrease in spite of an
156 increased plant N pool at high diversity (Fig. 1a-ii, 1b-ii), because some species enhance
157 their N-use efficiency in mixtures (Fargione et al. 2007, Fornara and Tilman 2009) or
158 legumes supply additional N through N-fixing (Palmborg et al. 2005). In CWs, this
159 situation may occur when microbial activities (e.g., nitrification and denitrification) are
160 limited due to the competition for N from plants (Matsushima and Chang 2007). (iii)
161 Neither plant N pool nor total N depletion has a detectable response to plant diversity
162 (Fig. 1a-iii, 1b-iii), for example due to the sampling effect (Gastine et al. 2003) or the
163 averaging effect (Engelhardt and Ritchie 2002). (iv) This class of responses is the most
164 distinct one, suggesting that other processes such as denitrification and leaching, in
165 addition to direct plant uptake, may account for the majority of N loss from the system
166 (Fig. 1b-iv). The first three cases (i, ii, iii) could occur under both N-poor (the major
167 pathway for N to enter the ecosystem is via N-fixation, e.g., grassland ecosystems, Fig.
168 1a) and N-rich conditions, however, the fourth one (iv) may only occur under high
169 external N supply (high anthropogenic N input, e.g., CW ecosystems, Fig. 1b) as tested
170 in the present study.

171 In microcosms simulating CWs, the amounts of N removal due to denitrification, N
172 accumulation in plant tissues and N adsorbed by the substrate can be quantified by MBA
173 (Kaye et al. 2006). However, because different processes may share the same reactant or
174 yield a common product, the MBA can only provide information on net rates of
175 production and consumption (Daufresne and Hedin 2005, Erler and Eyre 2010). As a
176 complementary approach, the isotopic signature of N species can reflect their origin and

177 allows the study of N processing in detail (Kendall et al. 2007). Therefore, SIA can be
178 used to identify each N transformation and the dominant N cycling routes in CWs. In the
179 present study, by combing MBA and SIA, we could study the cycle of N fluxes in
180 experimental CWs and assess the relative importance of the processes responsible for the
181 N removal. In addition, the N source we supplied in the wastewater was inorganic nitrate
182 (NO_3^-), which is less likely to be adsorbed by substrate, compared with ammonium (NH_4^+)
183 (Zhou et al. 2011). Thus, using NO_3^- as the sole N source can clarify the role of plants
184 and makes the results more interpretable.

185 **Experiment design**

186 The study site, located at the campus of Zhejiang University in Hangzhou City
187 (120º05' E, 30º18' N), southeast China, has a humid subtropical climate with an average
188 annual temperature of 18.7 °C and rainfall of 1,350 mm. The microcosms, which were
189 placed outdoor, simulated CWs and consisted of a porcelain column (length × width ×
190 height = 51 cm × 38 cm × 18 cm) filled with sand (particle diameter 2 mm) to a depth of
191 15 cm (1.43 g cm^{-3}). Prior to the experiment, the sand was washed with tap water to ensure
192 there were no nutrients left (pH = 7).

193 The simulated wastewater was Hoagland nutrient solution (Hoagland and Arnon
194 1950) with a minor modification (Table 1). Nitrate (NO_3^-) was the sole N source in the
195 influent, with a concentration of 112 mg L^{-1}. The stable nitrogen isotope ratio, $^{15}N/^{14}N$,
196 or $\delta^{15}N$, was 1.59‰. The simulated wastewater was supplied once every 10 days, from
197 June to late September (during the experiment the microcosms were not drained),
198 simulating the batch water operation mode of CWs (Faulwetter et al. 2009). Each
199 microcosm received 7 L simulated wastewater each time, and the water level was kept 2

200 cm above the sand surface. Tap water (municipal water) was supplied daily to compensate
201 for the water loss due to evapotranspiration. It should be noted that the amount of water
202 evaporating from each microcosm may not be the same, and rainwater also contained N
203 (the microcosms were placed outdoor), however, compared with the high N loading in
204 the simulated wastewater, N added by tap water and rainfall (total N concentration <3 mg
205 L^{-1}) was neglected. In addition, rainfall might wash away nutrients (N) in the microcosms,
206 but every microcosm was affected in the same way and the study area is characterized by
207 hot and dry climate from July to September, thus disturbance caused by rain was also
208 considered negligible in this study.

209 In June, seedlings of four plant species, *Phragmites australis* (Cav.) Trin. ex Steud.,
210 *Typha angustifolia* L., *Coix lacryma-jobi* L. and *Acorus calamus* L. with similar height
211 and vitality were transplanted into the sand substrate of the microcosms, ranging in
212 richness from one to four species. These species are common in subtropical wetlands in
213 China and are morphologically and functionally different, which allows them to access
214 resources in different ways. *Phragmites australis* is a mesophyte while the other three
215 species are hydrophytes. Both *P. australis* and *T. angustifolia* have well-developed
216 aerenchyma tissues while *C. lacryma-jobi* and *A. calamus* do not. More characteristics of
217 the four species are listed in Table S1. We planted vegetative propagules rather than seeds
218 in order to improve survival and shorten the duration of the experiment.

219 There were a total of 15 possible species-combination treatments (4 monocultures, 6
220 two-species mixtures, 4 three-species mixtures and 1 four-species mixture) which were
221 all included in the experiment. The experiment was arranged in a complete block design
222 with 3 replicate blocks, resulting in a total of 45 microcosms. Each microcosm had 12

plant individuals with an equal number of individuals assigned to each species. Germinating unplanted species were removed every week during the experimental period. During late vegetative phase, flower buds were manually removed as soon as they appeared to encourage more vegetative growth.

Sampling and measurements

Water samples were taken on the eighth day after the last supply of simulated wastewater. Before sampling, the water levels of all microcosms were kept constant to ensure that the same volumes of water were drained from each microcosm. Effluent (500 mL) was discharged at the bottom of the microcosms and then filtered through a membrane (pore size 0.45 μm, Xingya Experimental Materials, Shanghai). Concentrations of effluent NO_3^- and NH_4^+ were measured using a segmented flow analyzer (SAN plus, Skalar, the Netherlands). Concentrations of NO_3^- and NH_4^+ were then summed to give TIN concentration. NO_3^- and NH_4^+ were separated for $\delta^{15}N$ measurements by distillation with MgO and Devarda's alloy (Feast and Dennis 1996). In detail, a portion of the extract was steam-distilled with MgO to separate NH_4^+ on a steam distillation system; thereafter, the sample in the flask was distilled again after the addition of Devarda's alloy to separate NO_3^-. The liberated NH_3 was trapped using boric acid solution. The trapped N was acidified and converted to $(NH_4)_2SO_4$ using 0.02 mol L^{-1} H_2SO_4 solution. To prevent isotopic cross-contamination between samples, 25 mL of reagent-grade ethanol was added to distillation flasks and steam-distilled for 3 min between sample distillations (Hauck 1982). The H_2SO_4 solution containing NH_4^+ was then evaporated to dryness at 65 °C in an oven and analyzed for ^{15}N abundance using a stable isotope mass spectrometer (Delta V Advantage, Thermo Finnigan, Bremen,

246 Germany). The accuracy and reproducibility checked with reference materials IAEA-N2
247 (ammonium sulfate, +20.3‰) and IAEA-N3 (potassium nitrate, +4.6‰) were better than
248 0.3‰ and 0.2‰, respectively.

249 After the water sampling, all plant materials were harvested, cleaned, oven-dried at
250 65 °C for 72 h and measured for leaf, stem and root biomass. Because flower buds were
251 not allowed to develop, no reproductive parts (flowers, seeds and fruits) were present at
252 harvest. Plant N concentrations and foliar $\delta^{15}N$ values were measured in the harvested
253 plant material using an element analyzer linked to an isotope ratio mass spectrometer as
254 described above. Afterwards, the substrate of each microcosm was thoroughly
255 homogenized, and a 150 g fresh sample was weighed and extracted with 2 M KCl at a
256 ratio of 1:1.5 (water volume to substrate weight). NO_3^- and NH_4^+ concentrations were
257 then determined as described above. A subsample from each substrate sample was used
258 to determine soil water content. Soil bulk density of the microcosms was measured by the
259 coring method before harvesting plant belowground tissues.

260 **Calculations**

261 ***Mass-balance approach (MBA)***

262 Nitrogen mass balance in each microcosm was calculated as:

263 $$N_i = N_e + N_p + N_s + N_d \quad (1)$$

264 where N_i is the amount of influent N of each microcosm, calculated by multiplying
265 influent NO_3^- concentration (NO_3^- was the sole N source supplied in the wastewater) by
266 influent water volume; N_e is the amount of effluent N of each microcosm, calculated by
267 multiplying effluent TIN concentration (the sum of NO_3^- and NH_4^+) by effluent water
268 volume. Although NH_4^+ was not contained in the wastewater, it could be derived from N

recycling in the microcosms (i.e., mineralization of organic N, see "*Stable-isotope approach*"). Thus, N removal can be split up into two parts, removal in the form of NO_3^- or removal in the form of NH_4^+, and N removal referred to the sum of the two. N_p is the amount of N uptake by plants (plant N pool), calculated as Equation 2; N_s represents the amount of N accumulated in substrate (substrate adsorption), calculated by multiplying substrate TIN concentration by bulk density by volume; and N_d is the amount of N that was lost through denitrification, calculated as Equation 3.

Plant N pool (N_p) of each microcosm was calculated as:

$$N_p = N_{ps} + N_{pr} = PB_s \times PC_s + PB_r \times PC_r \quad (2)$$

where N_{ps} and N_{pr} are plant above- (shoots) and belowground (roots) N pool (mg m^{-2}), respectively; PB_s and PB_r are plant biomass of above- and belowground (g m^{-2}), respectively and PC_s and PC_r are N concentrations of above- and belowground (mg g^{-1}), respectively. The total plant N pool of each microcosm is the sum of N pool of individuals of each species.

The amount of N loss through denitrification was calculated as:

$$N_d = N_i - N_e - N_p - N_s \quad (3)$$

The contribution of denitrification to total N removal (fd) was calculated as:

$$f_d\ (\%) = N_d / (N_i - N_e) \times 100 \quad (4)$$

The contributions of other compartments (plants and substrate) to the total N removal were calculated following the same approach as Equation 4.

Stable-isotope approach (SIA)

In general, microbial N transformations discriminate against the heavy N isotope, leaving the substrate ^{15}N enriched and the product ^{15}N depleted (Fry 2006). For example,

292 in riparian buffer zones and CWs, decreasing effluent NO_3^- concentrations have been
293 successfully linked to an increasing accumulation of ^{15}N in NO_3^- because denitrifying
294 bacteria discriminate against the ^{15}N (Reinhardt et al. 2006, Erler and Eyre 2010). In
295 contrast, the N isotope fractionation caused by plants (e.g., assimilation) has often been
296 neglected, due to the small N isotope fractionation in plants (Robinson 2001, Choi et al.
297 2007, Erler and Eyre 2010). Meanwhile, because wetland plants change the oxidation
298 level of the soil by oxygen (O_2) release from roots (Armstrong 1979), in plant-inhabited
299 microcosms the occurrence and importance of dissimilatory nitrate reduction to
300 ammonium (DNRA) is generally not considered (Matheson et al. 2002).

301 Assuming that in the system denitrification is the only process resulting in the
302 fractionation of the N isotopes, the $\delta^{15}N$ of effluent NO_3^- (δ_e) can be calculated using the
303 Rayleigh distillation equation (Mariotti et al. 1988):

304 $$\delta_e = \delta_i + \varepsilon \ln(1 - f_d) \quad (5)$$

305 where δ_i is $\delta^{15}N$ of influent NO_3^-, ε is isotopic fractionation factor quantifying the degree
306 of fractionation of NO_3^- caused by denitrification (Robinson 2001) and f_d is the
307 contribution of denitrification to total N removal, obtained from Equation 4.

308 There are two possible relationships between the calculated (δ_e, obtained from
309 Equation 5) and observed (δ_o) effluent $\delta^{15}N$-NO_3^- concentrations: (1) $\delta_o \approx \delta_e$, this suggests
310 that the isotope fractionation of N is primarily due to microbial denitrification and effluent
311 NO_3^- is enriched with ^{15}N (Fig. 2a); (2) $\delta_o < \delta_e$, this suggests that other processes, in
312 addition to denitrification, cause N isotope fractionation. In other words, N recycling
313 occurs in the system (Fig. 2b). Plant species may feed internal N recycling: it assimilates
314 the supplied NO_3^- to form organic compounds (dead plant tissues and root exudates)

315 which could be subsequently transformed to NO_3^- again through mineralization and
316 nitrification. Because of the isotope fractionation against ^{15}N during the multistep N
317 processes, the newly produced NO_3^- from N recycling had lower $\delta^{15}N\text{-}NO_3^-$, partly
318 offsetting the increased effluent $\delta^{15}N\text{-}NO_3^-$ caused by denitrification (Choi et al. 2007).
319 As a result, δ_o would be lower than δ_e (the effluent contains both light and heavy NO_3^-).

320 **Statistical analysis**

321 Effects of species richness were examined by simple linear regression that identified
322 relationships between plant species richness and the response variables (effluent TIN
323 concentration, plant N pool, the amount of N loss via denitrification and substrate N
324 contents). One-way ANOVA with a Tukey's post hoc test was used to test for differences
325 in the above response variables as well as effluent $\delta^{15}N$ ($\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$)
326 among the 15 plant species-combination treatments. We also used ANOVA to separate
327 the effects of species richness from the effects of differences among species compositions
328 within richness levels on those response variables (Schmid et al. 2017). We used further
329 contrasts within species composition to test whether the presence of a particular species
330 in the experimental communities explained additional variation in the response variables
331 (Schmid et al. 2017). This same analysis was also repeated with independent-samples t-
332 tests to find differences between means of the response variables when a species was
333 present and when it was absent from the system. Bivariate Pearson correlations were used
334 to measure the correlations among pairs of variables involved in the N cycle. Prior to the
335 final analysis we tested wether residuals were normally distributed and had constant
336 variance across treatments, which was the case. All statistical analyses were done using
337 the software R 3.2.2 (R Core Team 2015).

RESULTS

Effects of species richness, identity and composition on N removal and multiple N-cycling processes in microcosms

Overall, increased plant species richness decreased effluent TIN concentration (Fig. 3a). The four-species mixture reduced effluent TIN concentration to the lowest level among all the 15 community treatments (transgressive under-depletion, Fig. 3a). In contrast, species richness was not significant for the three constituent N-cycling processes of N removal considered individually, i.e., plant uptake (plant N pool, Fig. 3b), denitrification (Fig. 3c) and substrate adsorption (substrate TIN concentration, Fig. 3d). There were significant differences in effluent TIN concentration among the 15 community treatments (Fig. S1a). However, species composition did not affect individual processes (Fig. S1b-d). ANOVA confirmed these results: effects of both species richness and species composition were significant for effluent TIN concentration (Table 2) with species richness explaining 16% and remaining species composition within richness explaining 48% of the total variation (Table 2). For the other three response variables, no significant effects were detected for species richness or remainig species composition within richness in the ANOVA (Table 2).

Although differences among the 15 community treatments were only significant for effluent TIN (Fig. S1), there were nevertheless significant contrasts when communities with a particular species present were compared with communities without that species. The presence of *C. lacryma-jobi* in the microcosm systems reduced effluent TIN by 34%, compared with its absence, while the presence of *P. australis* had a significant influence

in reducing effluent NH_4^+ concentrations. In contrast, the other two species had negligible effect on effluent N concentrations (Table 3). Total plant-N pools were increased when *C. lacryma-jobi* was present, but reduced when *P. australis* was present. The denitrification was increased by 44% in the presence of *P. australis* but decreased by 25% in the presence of *C. lacryma-jobi* (Table 3). None of the four species had a significant influence on substrate N concentrations (Table 3).

Effects of plant species on N transformation within microcosms

The stable isotope approach revealed that the concentration of $\delta^{15}N\text{-}NO_3^-$ in effluent differed among community treatments (Fig. 4). Communities with *A. calamus* had the lowest $\delta^{15}N\text{-}NO_3^-$ values while communities with *C. lacryma-jobi* or *T. angustifolia* had the highest (significant for *C. lacryma-jobi*; Table 3). Communities with *P. australis* were intermediate in effluent $\delta^{15}N\text{-}NO_3^-$ (Fig. 4). Species identity and composition had weaker effects on variation in effluent $\delta^{15}N\text{-}NH_4^+$ (Table 3 and Fig. 4, respectively). Nevertheless, monocultures of *P. australis* had considerably lower effluent $\delta^{15}N\text{-}NH_4^+$ than the other three monocultures (Fig. 4).

When comparing the observed effluent $\delta^{15}N\text{-}NO_3^-$ (δ_o) with model estimates (δ_e, Equation 5), monocultures of *C. lacryma-jobi* and *T. angustifolia* had similar δ_o and δ_e, i.e., $\delta_o \approx \delta_e$ (Fig. 5). By contrast, effluent $\delta^{15}N\text{-}NO_3^-$ measured for *P. australis* and *A. calamus* monocultures were lower than expected, i.e., $\delta_o < \delta_e$ (Fig. 5). When plotting δ_o against δ_e, communities including *C. lacryma-jobi* were close to the line $y = x$ ($\delta_o = \delta_e$) while communities without *C. lacryma-jobi* were far away (Fig. S2).

Interlinkage of different N processes and their contributions to N removal at different species richness levels

384 The integrated measure of N removal (effluent TIN concentration) from microcosms
385 was negatively correlated with the constituent N-cycle process of the plant N pool (Table
386 4), which was a major contributor to the N removal in the simulated CWs. At the same
387 time, effluent TIN concentration was negatively correlated with the concentration of
388 effluent $\delta^{15}N\text{-}NO_3^-$ (Table 4). Plant-N pool and total biomass were highly correlated. The
389 N-cycling process denitrification was negatively correlated with the other two N-cycling
390 processes, plant-N uptake (indicated by plant-N pool) and substrate absorption (indicated
391 by substrate TIN contents) (Table 4), i.e., when more N was accumulated in plants or
392 substrate less N was lost through denitrification.

393 All microcosms removed large amounts of N, ranging from 7315–8797 mg N per
394 microcosm (434–522 mg N m^{-2} d^{-1}) (Fig. S3). Plant uptake (above- and belowground N
395 pool) contributed the largest portion to the total N removal (21–72%) and denitrification
396 also accounted for a substantial N loss (20–50%). The role of substrate adsorption (the
397 amount of TIN in substrate) in N removal was relatively minor (3–20%) (Fig. S3).

398

399 **DISCUSSION**

400 **Transgressive under-depletion of wastewater N at high speices richness level**

401 Our results showed an overall positive effect of species richness on N removal in the
402 experimental CWs. In particular, the four-species mixture gave rise to a transgressive
403 "under-depletion" effect, i.e., the mean effluent TIN concentration of the four-species
404 mixture was lower than that of any monoculture. However, contrary to the results reported
405 in grassland ecosystems where "under-depletion" of soil N was attributed to excessive
406 plant uptake (Palmborg et al. 2005), in this study we did not find that the species mixtures

407 incorporated more N into their biomass than did monocultures. Thus, our data were
408 consistent with Fig. 1b-iv and supported hypothesis iv (see "Principle and methodology").
409 Meanwhile, polycultures did not outperform the most-efficient monoculture in either
410 denitrification or substrate adsorption. Hence, the "under-depletion" of wastewater N at
411 high richness level was surprising since none of the three constituent processes in the
412 mixed-species treatments outperformed the monoculture treatments.

413 This unexpected under-depletion of wastewater N in our study clearly demonstrated
414 a context dependency of biodiversity effects as: although species richness had negligible
415 effects on individual processes, it was far more important when multiple processes were
416 considered together. Because the response of a single process to biodiversity does not
417 equal that of the integrated ecosystem functioning, focusing on individual processes
418 might be misleading. Previous studies have proposed that the positive relationship
419 between species diversity and ecosystem functioning that consists of multiple processes
420 could happen because either all single processes increase with diversity (Byrnes et al.
421 2014), or species are complementary in terms of which processes they are important for
422 (Gamfeldt et al. 2008). Our findings confirmed the latter and suggested that the
423 complementary species could exhibit a nonlinear, rather than additive behavior (the
424 function of polyculture equals to the sum of the proportions of individual species function)
425 in their polycultures (Tilman 2014). In summary, our results indicated that: (1) effects of
426 plant diversity on N removal from N-rich ecosystems (e.g., CWs) should be investigated
427 by integrating multiple processes; (2) when multiple processes were considered, their
428 resulting ecosystem functioning could not be simply expressed as the sum or the average
429 of the constituent processes. Finally, it should be noted that in our study the integrated

ecosystem process N removal was measured directly and independently as TIN rather than by simply combining the three constituent processes via calculation to obtain a multifuncitonality measure. The latter is the approach that has typically been used in previous multifunctionality studies (e.g. Isbell et al. 2011, Perkins et al. 2015, Meyer et al. 2018, Le Bagousse-Pinguet et al. 2019).

Defining plant functional types based on species impacts on N transformation and cycling

Overall species-richness effects are expected to result from different species contributing differently to ecosystem functioning. Thus, our species might be assigned to different functional types with regard to their specific effects on N-cycle processes studied within the microcosms. Each plant species may have a distinct nutrient uptake rate and specific growth pattern in nature. Furthermore, the activity and relative abundance of rhizosphere microbial communities can be species-dependent (Garbeva et al. 2004, Hartmann et al. 2009). Therefore, species may differ in their behaviors that impact N transformation in CWs.

In this study, the high capacity of *C. lacryma-jobi* for N uptake resulted in an overall high N-removal efficiency. More N was fixed in its biomass (the highest amount among the four species) and only a small amount of organic N seemed to have been exuded by the roots (thus weak N recycling, $\delta_o \approx \delta_e$). In contrast, noticeable N recycling occurred in the rhizosphere of *P. australis* and *A. calamus* ($\delta_o < \delta_e$). While *P. australis* is generally regarded as a competitive species in wetlands (Vymazal and Kropfelova 2005), we found that *P. australis* had the lowest biomass production in our experimental microcosms (Fig. S4), probably due to its preference for ammonium over nitrate (Tylova et al. 2008; Table

3). *Phragmites australis* has been observed to show a pronounced tolerance towards high NH_4^+ supply (Tylova et al. 2008). Because NO_3^- was the sole N source we provided, the growth and competitive ability of *P. australis* seemed to have been compromised. This preference for ammonium may also result in low effluent $\delta^{15}N\text{-}NH_4^+$ (Fig. 4), which was readily converted from organic N and was assimilated by *P. australis* rapidly before being nitrified (thus little fractionation).

According to their different impacts on N cycling (which N transformation processes were predominant in their rhizosphere) in the microcosm systems, we divided the four species into three functional types (Fig. 6). *Coix lacryma-jobi* was classified as TI: a high level of N uptake but a low level of denitrification, and unnoticeable N recycling; *Acorus calamus* was classified as TII: modest N uptake and denitrification, but significant N recycling; and *Phragmites australis* was grouped into TIII: a low level of N uptake but high levels of denitrification and N recycling, and a strong preference for ammonium (Fig. 6). Since there were no statistically significant parameters found for *Typha angustifolia*, this species may not belong to any of the above types.

Effect of plant diversity on N removal in relation to competition and complementarity of different functional types

The nature of the effect of biodiversity on ecosystem functioning depends on the direct species contributions (trait dependent) or the role of coexistence mechanisms (Meyer et al. 2018). As discussed above, we might expect that the mixtures of TI (rapid N uptake) and TIII (great denitrification) would lead to a high N removal efficiency due to complementarity in N cycling processes. However, the “under-depletion” was not observed in their mixtures (Fig. S1a). Other two-species mixtures, such as TI × TII, and

TII × TIII, did not achieve the "under-depletion effect" either. Therefore, the presence of two functionally dominant species for individual processes did not guarantee that the integrated ecosystem functioning was sustained at a high level.

Given that NO_3^- was the sole N source available to plant uptake, TI may have a strong competitive advantage over TII and TIII. When *C. lacryma-jobi* was present in communities, leaf N concentrations of other co-existing species decreased (Fig. S5), suggesting that the nutrient acquisition of neighboring plants might have deteriorated. Thus, *C. lacryma-jobi* was a "nasty neighbor" that limited the accessibility to essential nutrients for a neighboring species, but not itself (Daufresne and Hedin 2005). This strategy may inhibit the activities of co-existing species and promote competitive exclusion. On the other hand, although *P. australis* had a great potential of denitrification, the preference for NH_4^+ may have constrained its growth. However, when *P. australis* was grown together with *A. calamus*, the demand of *P. australis* for NH_4^+ may have been satisfied because NH_4^+ was produced during the N recycling by *A. calamus* (*A. calamus* itself did not consume a large amount of NH_4^+, Table 3). Therefore, *A. calamus* could be considered a "nice neighbor" (Daufresne and Hedin 2005), because it increased the nutrient availability to its neighbor. Biomass production of the *P. australis*/*A. calamus* bi-culture was much greater than that of the *P. australis* monoculture (Fig. S4).

In summary, the under-depletion of N depended on the coexistence of different functional types (a type with high N uptake, TI; a type with high denitrifying potential and high NH_4^+ preference, TIII and a type that could provide additional NH_4^+, TII). The results of ANOVA supported our speculations as there were significant interaction effects between *P. australis* (TIII) and *A. calamus* (TII), and between *P. australis* (TIII), *A.*

499 *calamus* (TII) and *C. lacryma-jobi* (TI) on effluent TIN concentration (Table S2). As the
500 number of ecosystem processes increases, the number of functionally different species
501 required to sustain the ecosystem functioning may also increase. No single species (or
502 single functional type of species) may sustain full functioning of the ecosystem, which
503 therefore is increased by plant diversity. We acknowledge that our experiment ran for a
504 relatively short period (four months) because the peak season of the four plant species
505 runs from June through September. A multi-year experiment would be needed to
506 corroborate our results before they will eventually be applicable to full-scale CWs.
507
508 **Conclusions and management implications**
509 Our results demonstrated a transgressive "under-depletion" of N at high species-richness
510 level. Species richness enhanced integrated ecosystem multifunctioning as measured by
511 N removal, although it did not significantly affect the constituent processes (plant uptake,
512 denitrification and substrate adsorption) when tested individually. The significant effect
513 of species richness on multifunctionality must therefore have been caused by
514 asynchronous responses of individual processes to species richness. This result is of
515 practical importance in CWs, where various rather than a few dominant processes can
516 contribute to nutrient removal. We suggest that in CWs high treatment efficiency can be
517 achieved by using synergistic interactions among species through (1) assembling plant
518 species belonging to different but complementary functional types (for N transformation
519 and cycling), (2) supplying nutrients intermittently in order to maintain a heterogeneous
520 nutrient habitat facilitating the coexistence of different functional types and (3) harvesting
521 biomass to remove N that is sequestrated in plant tissue, thereby also relieving plants from

522 competition for space and light. Because species with greater biomass production are
523 more effective in reducing effluent N, this should also be a criterion for species selection
524 for wastewater treatment.
525
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529
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686

Tables

TABLE 1. Contents of nutrient components used in the simulated wastewater (modified Hoagland nutrient solution).

Macroelements	Contents ($g\ L^{-1}$)	Microelements	Contents ($mg\ L^{-1}$)
KNO_3	1.46	H_3BO_3	2.86
$Ca(NO_3)_2 \cdot 4H_2O$	1.13	$MnCl_2 \cdot 4H_2O$	1.81
$CaCl_2 \cdot 2H_2O$	0.50	$ZnSO_4 \cdot 7H_2O$	0.22
KH_2PO_4	0.14	$CuSO_4 \cdot 5H_2O$	0.08
$MgSO_4 \cdot 7H_2O$	0.49	$H_2MoO_4 \cdot 4H_2O$	0.09
KCl	0.09	$FeSO_4 \cdot 7H_2O$	5.56
		Na_2EDTA	7.44

692 **TABLE 2.** Summary of the ANOVA for effluent TIN, plant N pool, denitrification and

693 substrate TIN (total inorganic nitrogen). SS% are the percentages of between-microcosm

694 variation explained. Significant *P*-values are printed in bold.

Source	*df*	SS	MS	%SS	F	*P*	*df*	SS	MS	SS%	F	*P*
	Effluent TIN (mg N m^{-2} d^{-1})						Plant N pool (mg N m^{-2} d^{-1})					
Block	2	5937	2968	14	9.12	**<0.001**	2	6946	3473	2	0.41	0.669
Species richness	1	6889	6889	16	21.17	**<0.001**	1	9453	9453	3	1.11	0.301
Species composition	13	19889	1530	48	4.70	**<0.001**	13	87982	6768	26	0.79	0.660
Residual	28	9111	325	22			28	238521	8519	69		
	Denitrification (mg N m^{-2} d^{-1})						Substrate TIN (mg N m^{-2} d^{-1})					
Block	2	24738	12369	7	1.59	0.221	2	1193	597	7	1.67	0.206
Species richness	1	1626	1626	1	0.21	0.651	1	1160	1159	7	3.25	0.082
Species composition	13	125620	9663	34	1.24	0.302	13	5408	416	30	1.17	0.351
Residual	28	217503	7768	58			28	9978	356	56		

695

696

TABLE 3. Data summary of different forms of nitrogen in effluent, plant biomass, substrate and denitrification in the presence and absence of the four species. Values are means ± 1 SE. TIN, total inorganic nitrogen. Asterisks indicate statistically significant differences among means when a particular species is present *vs.* absent from communities. Values in boldface are significant at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Parameter		*P. australis*	*T. angustifolia*	*C.lacryma-jobi*	*A. calamus*
Effluent					
TIN (mg L^{-1})	Presence	184.8±18.2	154.1±16.5	**138.0±16.8****	164.3±15.7
	Absence	157.6±15.5	189.8±17.0	**210.0±13.0**	179.0±18.6
Nitrate (mg L^{-1})	Presence	166.7±17.7	133.3±16.0	**115.7±15.9*****	144.1±15.1
	Absence	133.7±14.8	168.7±16.8	**190.7±12.6**	157.4±18.4
Ammonium (mg L^{-1})	Presence	**17.8±2.0***	20.4±2.2	22.4±2.4	19.9±1.9
	Absence	**24.0±2.22**	21.1±2.2	18.9±1.8	21.6±2.5
Nitrate $\delta^{15}N$ (‰)	Presence	2.6±1.9	4.9±1.9	**9.1±1.7*****	4.2±2.2
	Absence	5.4±2.2	2.7±2.3	**-1.1±1.7**	3.5±1.8
Ammonium $\delta^{15}N$ (‰)	Presence	-3.3±1.9	-4.0±1.9	-3.6±2.3	-2.2±2.0
	Absence	-2.2±2.2	-1.5±2.1	-1.8±1.5	-3.5±2.1
Plant N pool					
Total (g m^{-2})	Presence	**19.7±1.7***	21.4±1.6	**23.7±1.3***	21.8±1.6
	Absence	**22.8±1.3**	20.9±1.6	**18.2±1.7**	20.5±1.5
Aboveground (g m^{-2})	Presence	12.8±1.4	14.1±1.3	**15.8±1.1***	13.8±1.3
	Absence	14.6±1.2	13.0±1.4	**11.1±1.4**	13.4±1.4
Belowground (g m^{-2})	Presence	6.9±0.9	7.3±0.9	7.9±1.0	8.0±1.0
	Absence	8.3±1.0	7.8±0.9	7.1±0.8	7.0±0.9
Denitrification (g N m^{-2})	Presence	**19.2±1.6***	17.8±1.7	**13.8±1.5***	17.2±1.7
	Absence	**13.3±1.3**	15.9±1.8	**18.5±1.8**	16.6±1.7
Substrate					
TIN (mg kg^{-1})	Presence	14.2±2.3	14.4±2.0	18.5±2.3	16.5±3.1
	Absence	20.6±3.5	20.7±3.8	15.8±3.8	18.3±2.9
Nitrate (mg kg^{-1})	Presence	5.4±1.2	4.4±0.7	5.6±1.2	4.7±0.9
	Absence	5.4±1.0	6.6±1.5	5.2±1.0	6.3±1.4
Ammonium (mg kg^{-1})	Presence	11.4±2.2	11.4±2.1	13.0±1.4	12.3±2.4
	Absence	15.9±2.7	15.9±2.8	14.1±3.4	14.7±2.5

705 **TABLE 4.** Correlation matrix of parameters related to the N cycle in the microcosms.
706 Both correlation coefficients (top right) and level of significance (*P* value, bottom left)
707 are shown. Number of observations are shown in parentheses. *** $P < 0.001$, ** $P < 0.01$,
708 * $P < 0.05$. TIN, total inorganic nitrogen, the sum of nitrate and ammonia concentrations.

709

	Effluent TIN	Plant N pool	Plant biomass	Denitrification	Substrate TIN	$\delta^{15}N\text{-}NO_3^-$	$\delta^{15}N\text{-}NH_4^+$
Effluent TIN		**-0.323* (45)**	**-0.389** (45)**	-0.091 (45)	0.145 (45)	**-0.468** (37)**	0.236 (42)
Plant N pool	0.041		**0.646*** (45)**	**-0.787** (45)**	-0.028 (45)	0.309 (37)	0.022 (42)
Plant biomass	0.008	<0.001		**-0.624** (45)**	-0.097 (45)	**0.400* (37)**	-0.101 (42)
Denitrification	0.550	0.001	0.001		**-0.363* (45)**	-0.128 (37)	-0.291 (42)
Substrate TIN	0.341	0.853	0.527	0.014		0.257 (37)	**0.480** (42)**
$\delta^{15}N\text{-}NO_3^-$	0.003	0.063	0.014	0.450	0.030		-0.060 (35)
$\delta^{15}N\text{-}NH_4^+$	0.133	0.888	0.524	0.061	0.001	0.730	

710

Figure legends

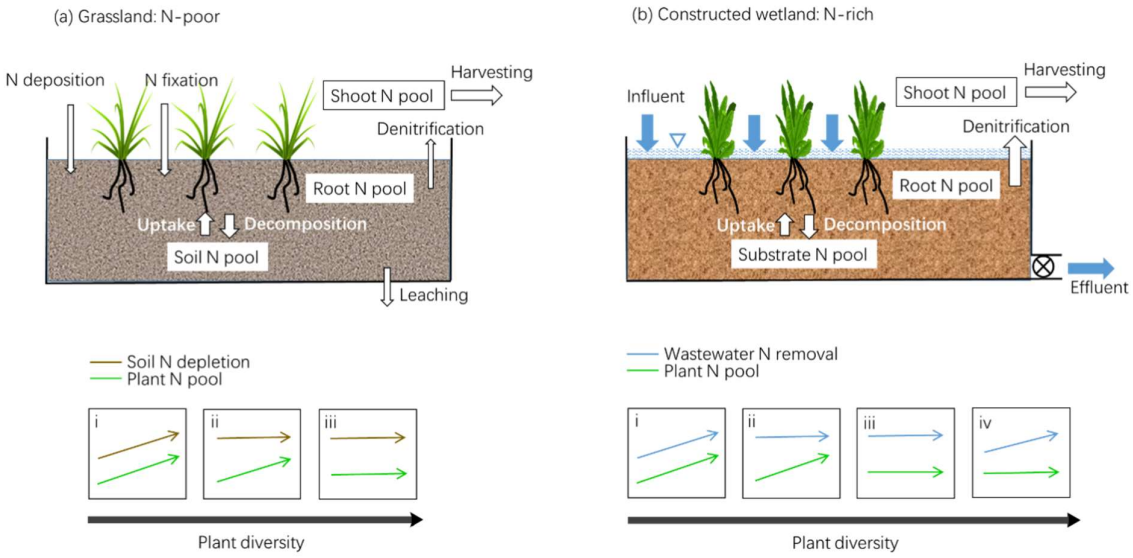
FIG. 1. Schematic diagram showing the major N flows in grassland (a) and constructed wetland ecosystems (b). Width of the arrows reflects the magnitude of the fluxes. All possible responses of soil-N depletion, wastewater-N removal and plant-N pool to increasing plant-species richness are shown in graphs i-iv (see "Principle and methodology" in "MATERIALS AND METHODS").

FIG. 2. Conceptual models for N removal with (a) and without (b) internal N recycling in the CWs. Solid black arrows represent N fluxes whilst dashed arrows represent diffusion. Ovals represent N reservoirs and their isotopic compositions (^{14}N and ^{15}N) are illustrated by different colors. The $\underline{N}$ represents the ^{15}N labelled nitrogen moving through the system. ε (fractionation factor, ‰) symbolized the degree of isotope fractionation during a reaction. ON, organic nitrogen. Note that the proportions of ^{15}N to ^{14}N in pie diagrams are not the actual values.

FIG. 3. Relationships between species richness with effluent TIN (a), plant N pool (b), the amount of N loss via denitrification (c) and substrate TIN (d). (a) is considered as an integrative measure of ecosystem multifunctioning, (b)-(d) are three key individual processes contributing to (a). Every circle represents a microcosm. Open triangles represent the mean of each community treatment. Values for triangles are means ± SE. Labels next to the means of monoculture treatments are species abbreviations (*Pa*, *Phragmites australis*; *Ta*, *Typha angustifolia*; *Cl*, *Coix lacryma-jobi*; and *Ac*, *Acorus calamus*). The dotted lines are the highest or lowest values in the monoculture. TIN, total inorganic nitrogen, the sum of nitrate and ammonia concentrations.

733 **FIG. 4.** Effluent $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ of different community treatments. Species
734 abbreviations are *Pa***,** *Phragmites australis*; *Ta*, *Typha angustifolia*; *Cl*, *Coix lacryma-jobi*;
735 and *Ac*, *Acorus calamus*. ANOVA tested for significant differences among treatment
736 manes. Values are means ± SE.
737 **FIG. 5.** Comparisons of the calculated and observed values of effluent $\delta^{15}N\text{-}NO_3^-$.
738 Species abbreviations are *Pa*, *Phragmites australis*; *Ta*, *Typha angustifolia*; *Cl*, *Coix*
739 *lacryma-jobi*; and *Ac*, *Acorus calamus*. Values are means ± SE. * indicate significant
740 difference between calculated and observed $\delta^{15}N$ values at $P < 0.05$.
741 **FIG. 6.** Multiple N processes in the microcosm systems. Black arrow lines indicate
742 expected pathways for N removal from the system. Grey arrow lines indicate N recycling
743 in the system. Species (the emoticons) were divided into three functional types according
744 to their impacts on N cycling: TI (*Coix lacryma-jobi*, in yellow), TII (*Acorus calamus*, in
745 orange) and TIII (*Phragmites australis*, in blue). Species contribute differently to each N-
746 cycling process. Happy emoticons indicate that species have a positive effect on a given
747 process and sad emoticons indicate that species have no particular effect on the process.
748 Pw, pathway; Pw A, NH_4^+ production (mineralization); Pw B, NH_4^+ uptake; Pw C, NO_3^-
749 uptake; Pw D, denitrification.
750

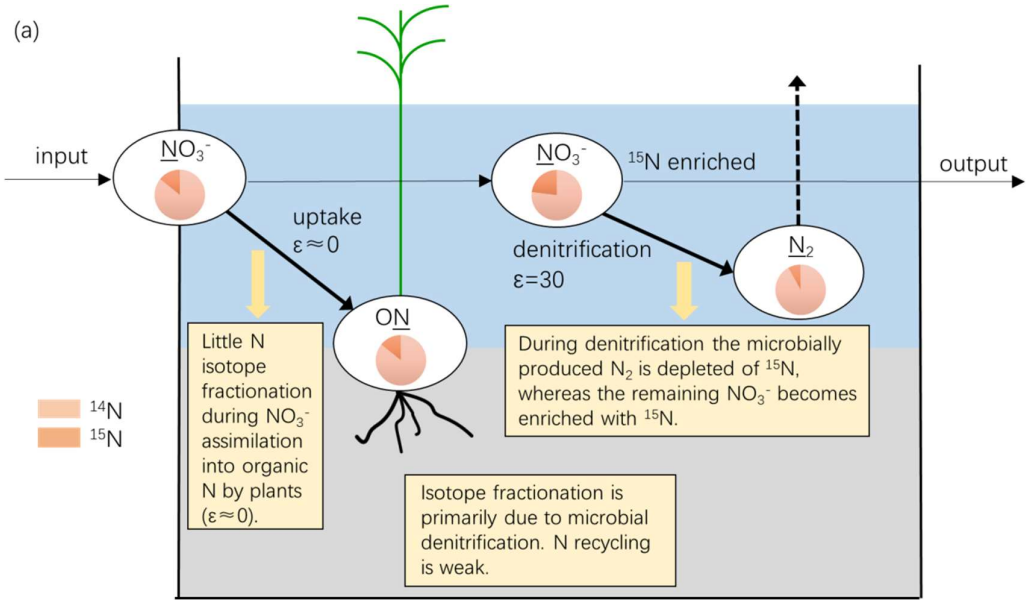
751 **FIG. 1.**



752

753

754 **FIG. 2.**



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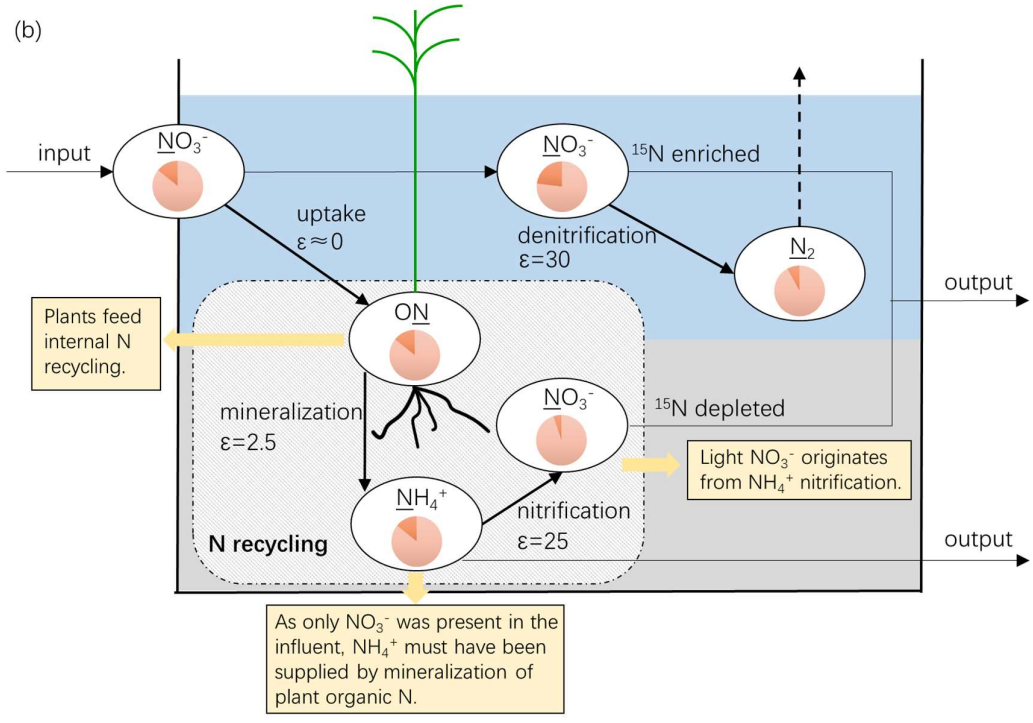
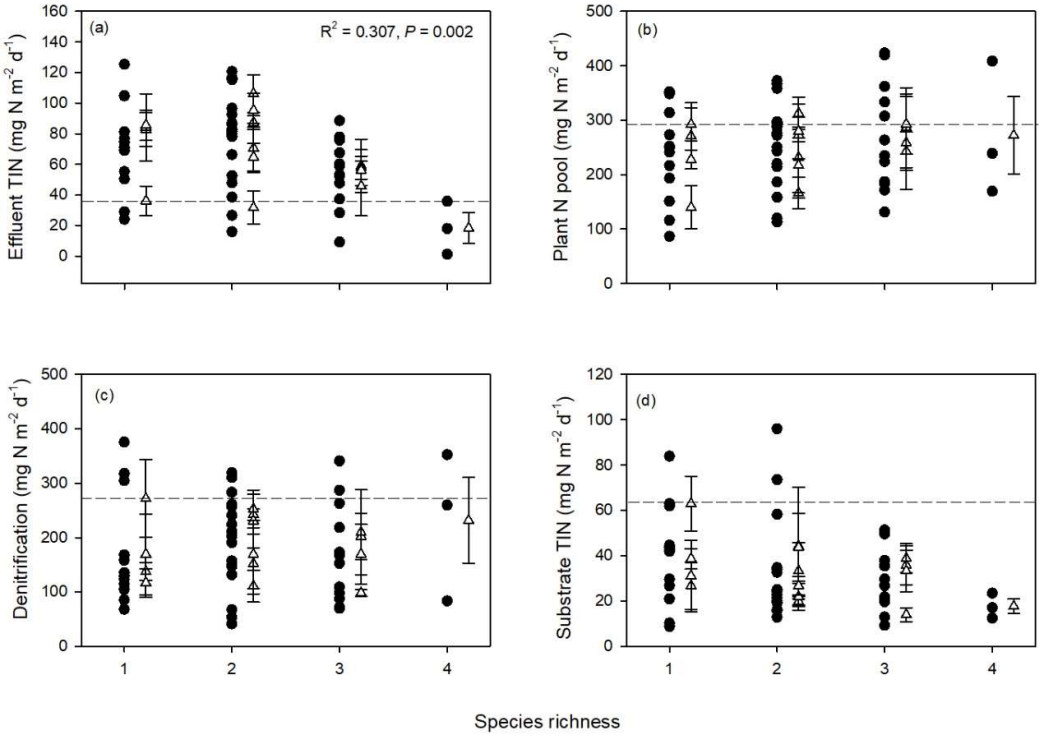
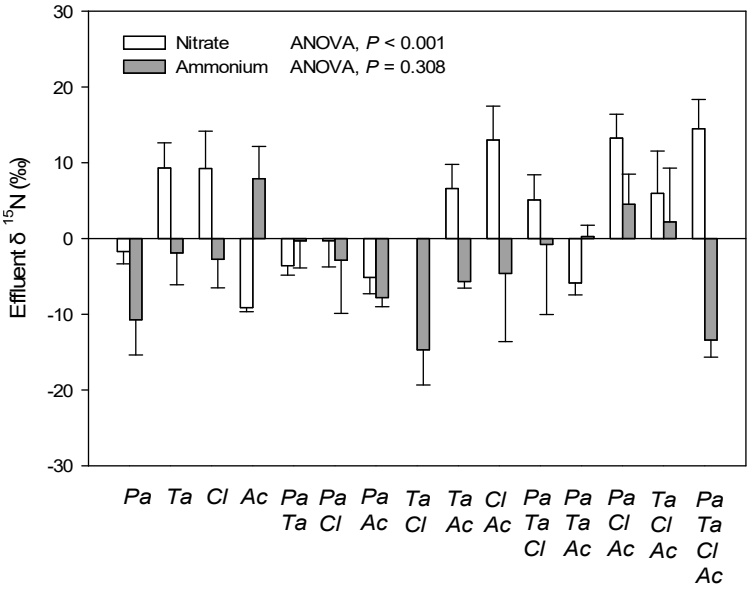


757

FIG. 3.

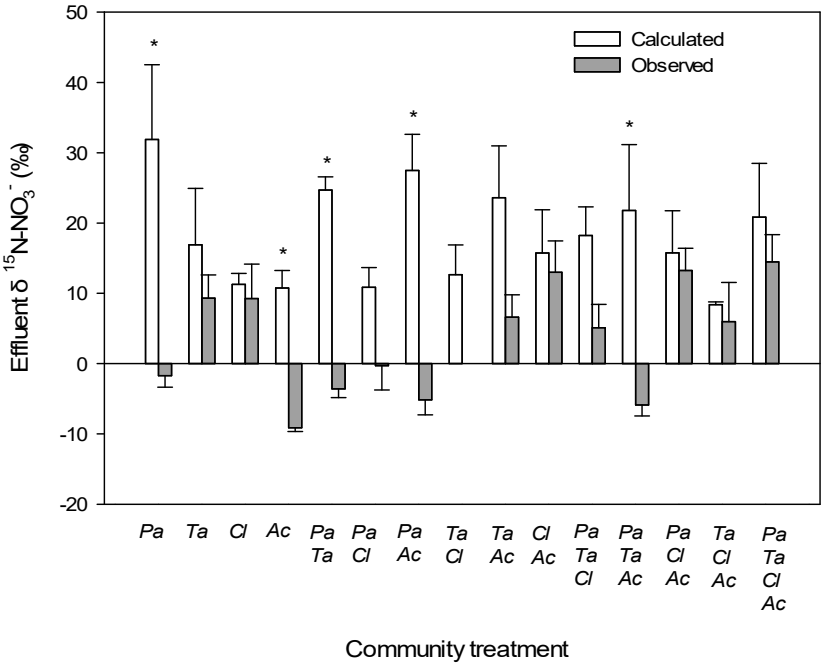

(a)
R^2 = 0.307, P = 0.002
Effluent TIN (mg N m^{-2} d^{-1})
(b)
Plant N pool (mg N m^{-2} d^{-1})
(c)
Denitrification (mg N m^{-2} d^{-1})
(d)
Substrate TIN (mg N m^{-2} d^{-1})
Species richness

761 **FIG. 4.**



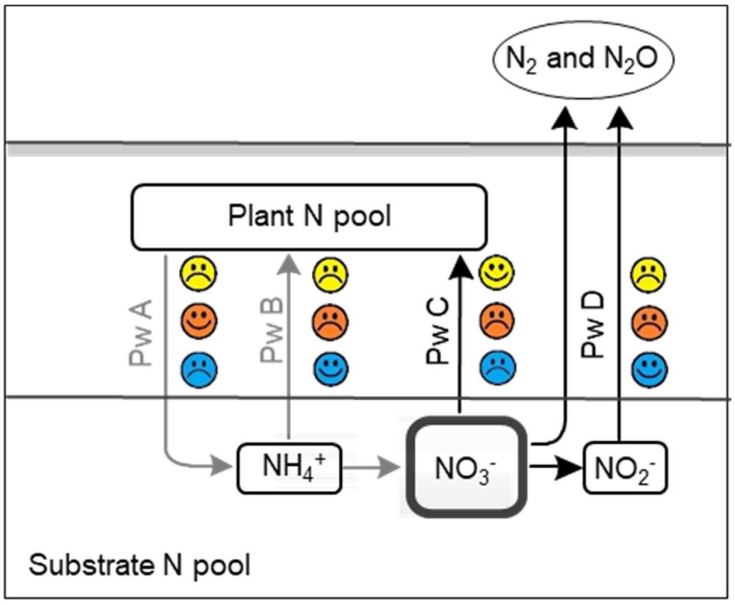
762
763

764 **FIG. 5.**



765
766

767 **FIG. 6.**


N2 and N2O
Plant N pool
Pw A
Pw B
Pw C
Pw D
NH4+
NO3-
NO2-
Substrate N pool

768